

University of Groningen

A comparative study of Tam3 and Ac transposition in transgenic tobacco and petunia plants

Haring, Michel A.; Gao, Jie; Volbeda, Tjeerd; Rommens, Caius M.T.; Nijkamp, H. John J.; Hille, Jacques

Published in:
 Plant Molecular Biology

DOI:
[10.1007/bf00016137](https://doi.org/10.1007/bf00016137)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
 Publisher's PDF, also known as Version of record

Publication date:
 1989

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Haring, M. A., Gao, J., Volbeda, T., Rommens, C. M. T., Nijkamp, H. J. J., & Hille, J. (1989). A comparative study of Tam3 and Ac transposition in transgenic tobacco and petunia plants. *Plant Molecular Biology*, 13(2). <https://doi.org/10.1007/bf00016137>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

A comparative study of Tam3 and Ac transposition in transgenic tobacco and petunia plants

Michel A. Haring, Jie Gao, Tjeerd Volbeda, Caius M.T. Rommens, H. John J. Nijkamp and Jacques Hille

Free University, Dept. of Genetics, de Boelelaan 1087, 1081 HV Amsterdam, Netherlands

Received 29 September 1988; accepted in revised form 24 April 1989

Key words: Ac, Tam3, transgenic plants, transposition assay, transposon tagging

Abstract

Transposition of the *Anthirrinum majus* Tam3 element and the *Zea mays* Ac element has been monitored in petunia and tobacco plants. Plant vectors were constructed with the transposable elements cloned into the leader sequence of a marker gene. *Agrobacterium tumefaciens*-mediated leaf disc transformation was used to introduce the transposable element constructs into plant cells. In transgenic plants, excision of the transposable element restores gene expression and results in a clearly distinguishable phenotype. Based on restored expression of the hygromycin phosphotransferase II (HPTII) gene, we established that Tam3 excises in 30% of the transformed petunia plants and in 60% of the transformed tobacco plants. Ac excises from the HPTII gene with comparable frequencies (30%) in both plant species. When the β -glucuronidase (GUS) gene was used to detect transposition of Tam3, a significantly lower excision frequency (13%) was found in both plant species. It could be shown that deletion of parts of the transposable elements Tam3 and Ac, removing either one of the terminal inverted repeats (TIR) or part of the presumptive transposase coding region, abolished the excision from the marker genes. This demonstrates that excision of the transposable element Tam3 in heterologous plant species, as documented for the autonomous element Ac, also depends on both properties. Southern blot hybridization shows the expected excision pattern and the reintegration of Tam3 and Ac elements into the genome of tobacco plants.

Introduction

The isolation and characterization of maize transposable elements has led to the application of the cloned transposons as probes for the detection of mutated plant genes [6, 11, 25, 26, 27]. These transposon tagging experiments indicate that mutated plant genes are preferably isolated from lines which 1) carry mutations that confer a clearly distinguishable phenotype and 2) have a low copy number of the active transposable element in their

genome. However, until now, active transposons have been cloned only from *Antirrhinum majus* and *Zea mays* (for a review see Nevers *et al.* [25]), although transposon-like elements have been isolated from several other plant species [7, 14, 30, 34]. To extend the possibilities of transposon tagging to other plant species experiments have been performed to test the activity of the maize transposable element Ac in heterologous plant species. It has been shown that the maize Ac element can transpose when it is introduced into

Nicotiana tabacum, *Daucus carota*, *Arabidopsis thaliana*, *Solanum tuberosum* and *Lycopersicon esculentum* by *Agrobacterium*-mediated transformation [1, 19, 33, 35].

Genetic analysis of transposition has been simplified by the use of a phenotypic assay allowing direct selection of excision events [2]. When Ac excises from the leader sequence of the neomycin phosphotransferase (NPTII) gene, the expression of the gene is restored and transformed calli become kanamycin-resistant. No kanamycin-resistant calli are found when a Ds, dissociator, element (an Ac element with an inactivated transposase function) is tested [2, 21]. Subsequent molecular analysis of the resistant plants demonstrated the reintegration of the element. Therefore, it can be envisaged that transposon tagging might be applicable to plant species from which endogenous elements have not yet been isolated, by using heterologous transposons as tags. The frequency of Ac excision ranges from 28 to 60% in the different plant species analyzed and in most cases reintegration of the element at different locations in the genome has been observed. Furthermore, the low copy number of the introduced T-DNA will ensure effective recloning of the tagged gene.

As an alternative for the monocot Ac element we have studied Tam3, an *Antirrhinum majus* transposon, for its suitability as a molecular tag. The 3.5 kb Tam3 element was cloned from the *nivea* locus [31] and has been used to isolate the *pallida* locus from *A. majus* [24]. This dicot element has some properties that make it an interesting candidate for transposon tagging in heterologous plant species. First, it has been shown to transpose from marked, endogenous sequences and secondly it can be induced to excise with a 1000-fold higher frequency by a temperature down-shift [24]. As these properties are exhibited at different loci in *A. majus* it has been suggested that these are specific qualities of Tam3 [5]. We report the use of a phenotypic assay, based on restored hygromycin resistance or β -glucuronidase activity, to compare the excision of Tam3 in transgenic *Nicotiana tabacum* and *Petunia hybrida* plants and we compare the fate of Tam3 and Ac

in transgenic plants by subsequent molecular analyses.

Materials and methods

Construction of a Tam3 cassette

To reduce the flanking *pallida* sequences of Tam3 in pAJ-4 (a gift from Dr C. Martin, Norwich, described in [24]) we cloned a blunted *Nru*I/*Hind* III fragment in the *Sma* I site of pUC19, so that only 14 base pairs (bp) *pallida* sequence flanked Tam3 at its *Sma* I end (Fig. 1B). The other end of this clone was deleted by *Bal* 31 digestion [23], starting from the *Eco* RI site of pUC19. After digestion with *Hind* III and filling in, the deleted fragments were isolated from agarose gel and cloned into the *Hinc* II site of pUC19. In this way fragments inserted in one orientation would have *Bam* HI sites on either side. Using *Bam* HI and *Pvu* II digestion we selected appropriate deletions of Tam3. After M13 sequencing [29] of both ends, a clone was selected (pVUT22) that contained 28 bp of *pallida* flanking sequence at the *Pvu* II end of Tam3 (Fig. 1C). This *Bam* HI clone contained the intact Tam3 element, while another clone (pVUT62) contained a deletion of the terminal inverted repeat sequence of one end (Tam3 Δ IR, Fig. 1D). An internal deletion of pVUT22 was made by replacing the 1.5 kb *Cla* I-*Nco* I Tam3 fragment by plasmid pACYC 184 cut with *Sal* I/*Hind* III (Tam3 Δ transposase, pVUT42; Fig. 1E).

Phenotypic assay vectors for Tam3 and Ac

Into the *Bam* HI site of the expression cassette pRok1 [3] we cloned the hygromycin phosphotransferase (HPTII) gene (a 1 kb *Bam* HI fragment with a single ATG to improve the translation efficiency; van den Elzen, unpublished) and modified the resulting plasmid by filling in the *Bam* HI site between the HPTII gene and the nopaline synthase (nos) polyadenylation signal. After the cloning of plasmid pUC18, containing

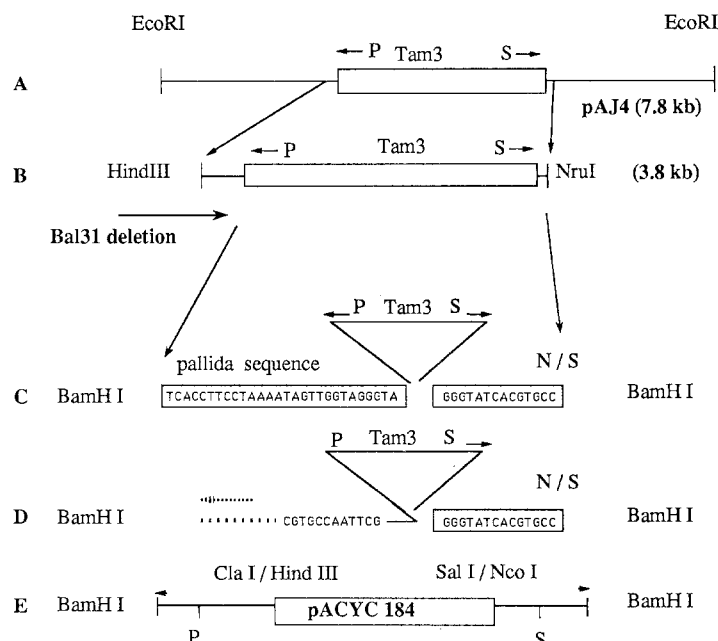


Fig. 1. Tam3 cassettes. Tam3 cassettes were derived from the *Eco*RI fragment of pAJ-4 fragment isolated from the *pallida* locus of *A. majus* (A). Bal31 deletions were carried out on the *Hind*III-*Nru*I subclone (B), yielding: (C) pVUT22, a 3.6 kb *Bam*HI fragment in pUC19, containing the intact Tam3 element flanked by 42 bp of *pallida* sequence, and (D) pVUT62 a 3.5 kb cassette with the *Pvu*II end of Tam3 deleted by 28 bp (Δ IR). By replacing the internal 1.5 kb *Cla*I/*Nco*I fragment of the Tam3 element from pVUT22 by pACYC184 an 5.2 kb element deleted in transposase function (Δ transposase) has been created, pVUT42 (E). Arrows indicate inverted repeats and N/S marks the *Nru*I/*Sma*I fusion of Tam3 and pUC19. The *Pvu*II (P) and *Sma*I (S) site of Tam3 are indicated to mark the orientation of the element.

the bases 2640 (*Sph*I)-6130(*Xho*I) from the T-DNA of pTiAch5 [12] in the *Hind*III site, the plasmid has been renamed pTT218 (Fig. 2A). The results summarized in Tables 1 and 2 confirmed that the expression of this reporter, the modified HPTII gene, was sufficient for selection.

Tam3 derivatives were inserted, all in the same orientation, in the *Bam*HI site between the CaMV 35 S promoter and the HPTII gene of plasmid pTT218. Plasmid pTT21820 contained an intact Tam3, pTT21806 contained Tam3 Δ IR and plasmid pTT21840 has Tam3 Δ transposase respectively (Fig. 2A). Tam3 derivatives were also inserted in the *Bam*HI site between the CaMV 35 S promoter and the β -glucuronidase (GUS) gene of plasmid pBI121 [19]. Plasmid pGT153 contained intact Tam3, while pGT113 contained Tam3 Δ IR and pGT114 contained Tam3 Δ transposase (Fig. 2B).

The 4.6 kb *Bss*HII Ac fragment from the *waxy-m7* allele [4] was equipped with *Bgl*II

linkers and cloned into the *Bam*HI site of pTT218, resulting in pTT224 (Fig. 3). After the removal of the terminal 200 bp *Bss*HII-*Bam*HI fragment the remaining 4.4 kb were cloned into pTT218, resulting in pTT228 (Δ IR). Insertion of plasmid pACYC184 into the *Xho*I site of pTT224 resulted in pTT229 (Δ transposase). These manipulations were carried out in *Escherichia coli* strain MH1 [13].

Plant transformation

The constructed binary vectors were conjugated to *Agrobacterium tumefaciens* LBA4404 [16] using the helper plasmid pRK2013 [10]. Leaf discs of *N. tabacum* cv. Petit Havana SR-1 [22] were infected by dipping in overnight culture *Agrobacterium* strains in LB-medium, while *P. hybrida* cv. Mitchell [9] leaf discs were incubated for 20 minutes in bacterial cultures diluted 1:100 in

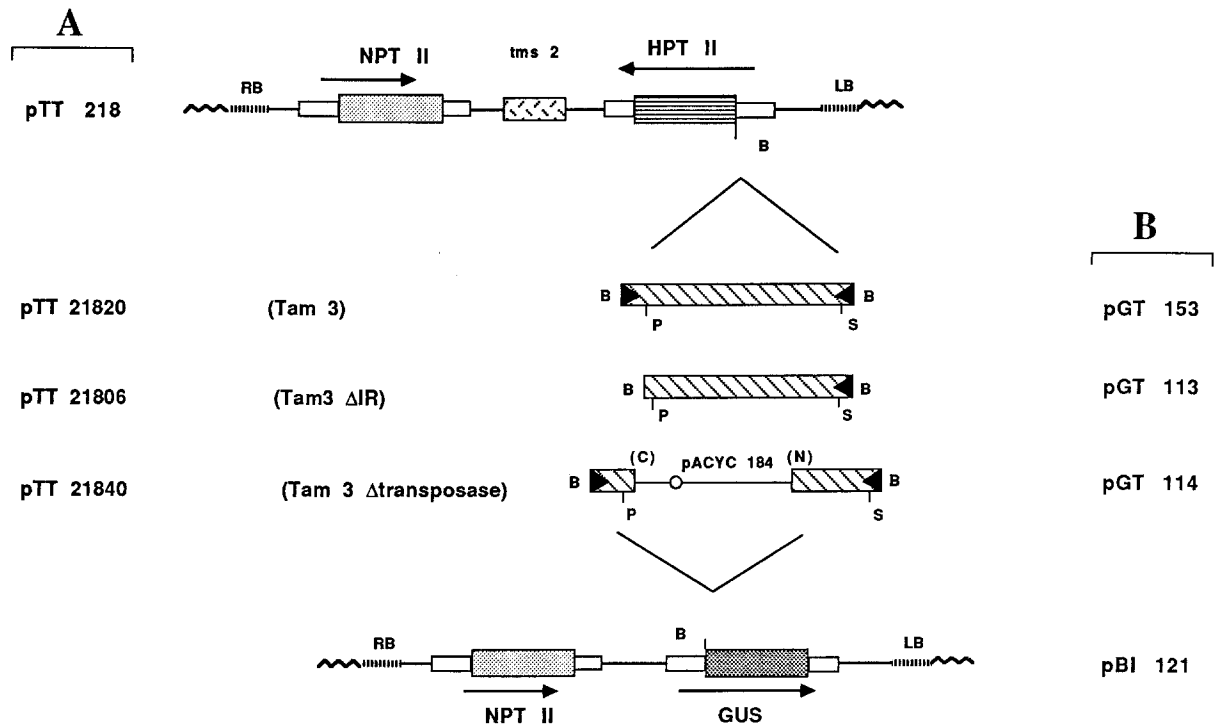


Fig. 2. Transposition assay vectors containing Tam3. A. Hygromycin resistance as the phenotypic marker. Binary vector pTT218 has both the NPTII and the HPTII gene and contains the *A. tumefaciens* T-DNA gene 2 (*tms-2*) as an additional marker. Tam3 derivatives inserted in the *Bam* HI site of PTT218 resulted in pTT21820 (active element), pTT21806 (deleted inverted repeat) and pTT21840 (inactivated transposase). B. β -glucuronidase activity as the phenotypic marker. The Tam3 derivatives were inserted into the *Bam* HI site between the CaMV 35 S promoter and the GUS gene of pBI121, resulting in the binary vectors pGT153 (deleted inverted repeat) and pGT114 (inactivated transposase). Direction of transcription is indicated by arrows and T-DNA borders are designated RB (right border) and LB (left border). Inverted repeats of the transposable elements are represented by black triangles. Restriction enzyme sites are abbreviated: B = *Bam* HI, C = *Cla* I, N = *Nco* I, P = *Pvu* II and S = *Sma* I.

MS-medium. Infected explants were dried briefly on filter paper and put on feeder layers of *Petunia* Albino Commanche suspension cells for two days. Transformed calli were selected on MS-medium containing carbenicillin (200 mg/l), vancomycin (200 mg/l), zeatin (2 mg/l) and kanamycin sulphate (150 mg/l) (all chemicals from Sigma). For the selection of hygromycin-resistant calli or shoots Hygromycin B was added to the medium in a concentration of 20 mg/l for petunia and 60 mg/l for tobacco.

GUS assays

GUS assays were performed on 100 mg leaf tissue from two-week-old shoots or greenhouse-

grown plants, as described by Jefferson *et al.* [19], using the substrate methyl umbelliferone glucuronide (MUG, Sigma). Enzyme activity was visualized by fluorescence of the product, MU, on a long-wavelength transilluminator.

Plant DNA isolation and Southern blot analysis

Total tobacco DNA was isolated as described [8] and 10 μ g of DNA was digested with 50–100 units restriction enzyme as described by the manufacturer (Biolabs Research Laboratories). After electrophoresis through a 1.2% agarose gel the DNA was blotted onto Hybond-N membrane (Amersham) with 10X SSC as transfer buffer. Hybridization was performed in 10% dextran sul-

Table 1. Tam3 excision in transgenic petunia and tobacco plants monitored by restoration of hygromycin resistance (A) and β -glucuronidase activity (B). The plasmids of the *Agrobacterium* strains used are described in Fig. 2. n.d. = not done.

		Tam3	Tam3 Δ IR	Tam3 Δ transp
A. Hygromycin reporter gene				
<i>Agrobacterium</i> strains	pTT218	pTT21820	pTT21806	pTT21840
<i>N. tabacum</i>				
Kan ^r calli	178	182	78	118
Hyg ^r calli	115	78	0	0
Frequency (%)	65	43	–	–
<i>P. hybrida</i>				
Kan ^r calli	78	105	59	54
Hyg ^r calli	43	23	0	0
Frequency (%)	55	22	–	–
B. GUS reporter gene				
<i>Agrobacterium</i> strains	pBI121	pGT153	pGT113	pGT114
<i>N. tabacum</i>				
Kan ^r	40	46	30	n.d.
GUS +	26	6	0	n.d.
Frequency (%)	65	13	–	–
<i>P. hybrida</i>				
Kan ^r	35	32	20	n.d.
GUS +	25	4	0	n.d.
Frequency (%)	71	13	–	–

phate, 1 M NaCl, 1% SDS and 200 μ g/ml denatured Salmon sperm DNA at 60 °C, using ³²P-dATP, random prime-labelled DNA fragments (with a specific activity of > 10⁸ cpm/ μ g). After 16 hours of hybridization the filters were washed down to 0.5X SSC, 0.1% SDS at 60 °C and autoradiographs were made with Kodak X-Omat AR films at – 70 °C on an intensifying screen.

Results

Construction of Tam3 cassettes and phenotypic assay vectors

For the construction of a Tam3 cassette, we deleted most of the *pallida* sequences from pAJ-4 [24], thereby removing all ATGs present in these flanking sequences. Plasmid pVUT22 (Fig. 1C),

Table 2. Ac excision in transgenic tobacco plant monitored by the restoration of resistance to 60 mg/l hygromycin in the medium. The frequency of calli resistant to 20 mg/l hygromycin is indicated in parenthesis. n.d. = not done.

		Ac	Ac Δ IR	Ac Δ transp
<i>Agrobacterium</i> strains	pTT218	pTT224	pTT228	pTT229
<i>N. tabacum</i>				
Kan ^r calli	158	163	100	103
Hyg ^r calli (60)	102	28	0	0
Hyg ^r calli (20)	131	58	n.d.	n.d.
Frequency (%)	65 (83)	17 (36)	–	–

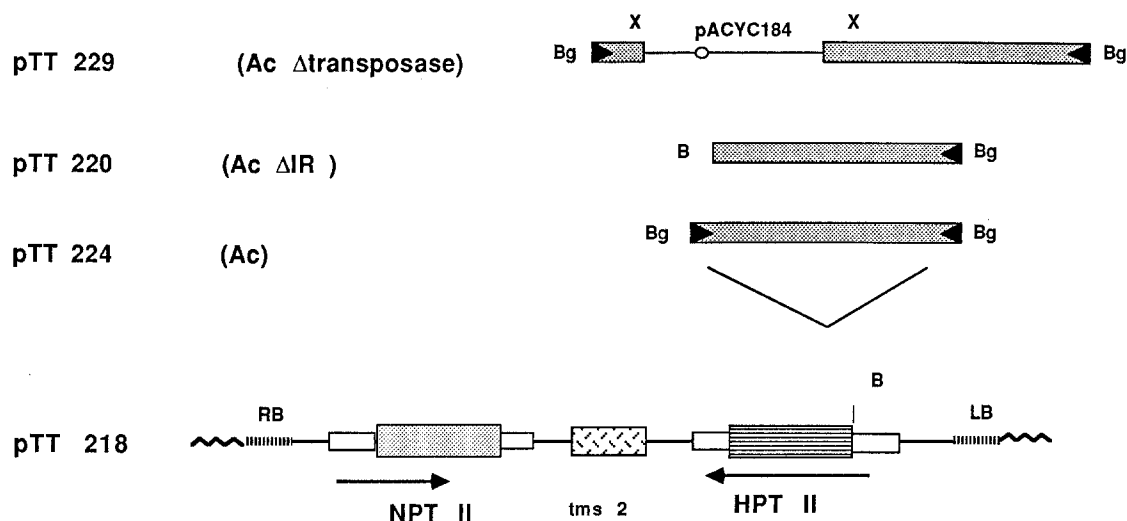


Fig. 3. Transposition assay vectors containing Ac. Cloning the Ac derivatives in the *Bam* HI site of pTT218 resulted in pTT224 (active element), pTT220 (deleted inverted repeat) and pTT229 (inactivated transposase). Direction of transcription is indicated by arrows and T-DNA borders are designated RB and LB. Inverted repeats of transposable elements are represented by triangles. Restriction enzyme sites are abbreviated: B = *Bam* HI, Bg = *Bgl* II and X = *Xho* I.

comprising the complete Tam3 element and flanked by 42bp *pallida* sequences, was the result of these manipulations (see Materials and methods). Because authentic transposition is dependent on both a functional transposase and intact inverted repeats, controls for the excision assays were constructed. We developed a Tam3 element with one of the TIR (terminal inverted repeat) sequences deleted, pVUT62 (Fig. 1D), which is not expected to be capable of excision (compare Tam1 [17]), and a Tam3 element from which a 1.5 kb internal fragment had been replaced by a plasmid, disturbing the putative transposase activity, but leaving the TIRs intact (Fig. 1E). All derivatives of Tam3 were inserted into the unique *Bam* HI site between the CaMV 35S promoter and the HPTII gene of the binary vector pTT218 (Fig. 2A). In order to have an additional and independent assay for Tam3 excision, we decided to use an assay without selective pressure, based on restoration of GUS gene expression. The binary vector pBI121 confers both kanamycin resistance and β -glucuronidase activity to plants [19]. Tam3 derivatives were cloned into the *Bam* HI site of the leader sequence of the GUS gene (Fig. 2B) resulting in the vectors pGT153 (Tam3), pGT1133 (Δ IR) and pGT114 (Δ transposase).

The 4.6 kb *Bss* HII fragment containing the Ac element flanked by 60 bp of waxy sequence from the *wx-m7* allele of maize was converted into a *Bgl* II fragment and cloned into the *Bam* HI site of pTT218 (Fig. 3). We also cloned deleted versions, that were created by removing the terminal *Bss* HII-*Bam* HI fragment, resulting in a deleted TIR (pTT228) and by the insertion of a plasmid into the *Xho* I site, localized in the transcribed region of the Ac element, resulting in inactivation of the transposase (pTT229).

Tam3 excision in transgenic plants

Excision of Tam3 in transgenic plants can be monitored by restoration of HPTII gene expression. *A. tumefaciens* strains harbouring the binary vectors described above were used to infect leaf discs of *N. tabacum* and *P. hybrida*. Kanamycin-resistant calli were grown for 2–3 weeks, cut in halves and transferred to fresh medium containing either kanamycin or hygromycin. After 3 weeks, the number of hygromycin-resistant calli was determined. The results of 3 independent experiments are summarized in Table 1.

Transformation of tobacco leaf discs with the

Agrobacterium strain containing plasmid pTT218 resulted in 65% hygromycin-resistant calli from the selected kanamycin-resistant calli (Tables 1A and 2). The expression of a second marker on the T-DNA is known to vary significantly in transgenic plants [20], which would account for the hygromycin-sensitive calli obtained from transformation experiments with pTT218. Three independent transformations with plasmid pTT21820, having Tam3 inserted in the HPTII gene, produced 78 hygromycin-resistant tobacco calli (43%) and 23 hygromycin-resistant petunia calli (22%). No hygromycin-resistant calli were detected amongst the calli transformed with pTT21806 (Δ IR) or pTT21840 (Δ transposase), suggesting that authentic Tam3 excision is monitored, because restoration of the HPTII gene is dependent on the presence of both TIRs and a functional transposase locus.

In order to have an independent assay for Tam3 excision, we decided to use a transposition assay without selective pressure based on restoration of GUS gene expression. In this case both petunia and tobacco were infected with *Agrobacterium* strains containing the vectors pBI121, pGT153 and pGT119. Leaves of shoots that had rooted on kanamycin-containing medium were used to determine the β -glucuronidase activity in a micro-assay using the fluorescent dye MU, which can be detected under UV light. Results of two tobacco transformation and two petunia transformation experiments are summarized in Table 1B. In 70% of the kanamycin-resistant shoots transformed with vector pBI121 we detected β -glucuronidase activity. When Tam3 was introduced into the leader sequence of the chimaeric GUS gene (pGT153), 6 GUS-positive plants were found amongst the 46 transformed tobacco plants analysed (13%). The results of the experiments with petunia plants indicated that Tam3 excises also in 13% of the transgenic plants analysed (Table 1B). No restoration of the GUS gene expression was detected, when Tam3 with a deleted inverted repeat was introduced into the leader sequence of the GUS gene (pGT113) and used to transform plants.

Ac excision in transgenic tobacco plants

Similar results were obtained when Ac was used instead of Tam3 (Table 2). In these experiments 65% of the 158 pTT218-transformed tobacco calli turned out to be resistant to 60 mg/l hygromycin. However considering the orientation of the HPTII gene in pTT218, close to the left-border sequence, it could be expected that plant regulatory sequences might influence the expression of this marker gene (so-called position effects). Therefore, we tested part of the calli on 20 mg/l hygromycin simultaneously. The number of hygromycin-resistant calli, transformed with pTT218 increased to 131 (83%), when this level of selection was applied. Disruption of the HPTII gene by Ac, as in plasmid pTT224, resulted in 17% hygromycin-resistant transformants. At a concentration of 20 mg/l hygromycin 58 of the calli tested were resistant (36%). Transformation with pTT220 (Δ IR) and pTT229 (Δ transposase) did not result in any hygromycin-resistant calli, suggesting that Ac excision is monitored, because restoration of the HPTII gene expression is apparently dependent on the presence of both TIRs and transposase.

Molecular analysis of Ac transposition

To establish that the restored HPTII gene expression resulted from Ac excision we analysed the DNA of hygromycin-resistant plants. An *Eco* RI digest should be able to distinguish a HPTII gene interrupted by Ac from a restored HPTII gene when hybridized with a CaMV probe (Fig. 4C). DNA isolated from tobacco plants, with a hygromycin-resistant phenotype, obtained by transformation with the vector pTT224, was digested with *Eco* RI, blotted onto Hybond-N and probed with the 0.9 kb *Eco* RI-*Bam* HI CaMV fragment. In five independent transformants a 1.1 kb fragment was detected (Fig. 4A, lanes 1–5), representing the fragment carrying the empty donor site. As expected this fragment is slightly larger than the original pTT218 fragment (Fig. 4A, lane 7), because 70 bp *waxy* sequence remains

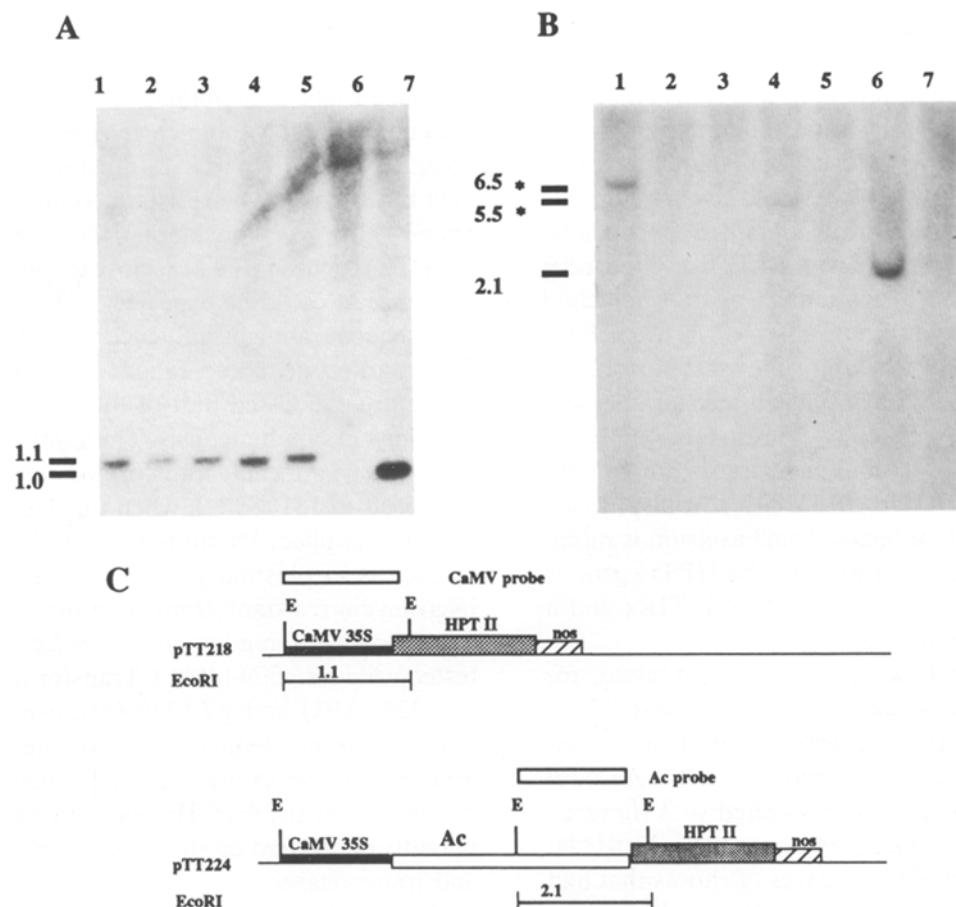


Fig. 4. Southern blot analysis of Ac excision. 10 μ g total DNA from 5 different pTT224-transformed plants, with a hygromycin-resistant phenotype (lanes 1–5), with a sensitive phenotype (lane 7) and DNA of a pTT218 transformed plant (lane 6) was digested with *Eco* RI, run on agarose gel and blotted onto Hybond-N membrane. Part A reveals the hybridization of the CaMV fragment (as described in C) to the filter, while the results of the hybridization to the Ac probe (as described in C) is shown in panel B. Fragment sizes are in kilobase pairs and new Ac insertions are indicated (*). The partial physical map is given in C) with the expected size of the generated fragments and the fragments used as probes.

after excision. No fragment of this size can be detected in the hygromycin-sensitive plant (Fig. 4A, lane 6).

Probing the same blots with Ac should provide the evidence that the element has integrated into the plant genome at new positions. The hybridization with the Ac fragment yielded fragments differing from the original 2.1 kb *Eco* RI fragment of pTT224 (Fig. 4B, lane 6) in the DNA of three plants (Fig. 4B, lanes 1 and 4), while two plants did not reveal any distinct bands. The integration of Ac into the genome of *N. tabacum* after excision and the detection of the fragment carrying the

empty donor site in hygromycin-resistant plants, provides the evidence that the phenotypic assay results correlate directly with the molecular data on transposition of Ac.

Tam3 excision at the molecular level

A similar analysis as described above was performed to analyse the molecular structure of the HPTII gene in hygromycin-resistant and -sensitive plants obtained from transformation with pTT21820. As noted above, an *Eco* RI digest of

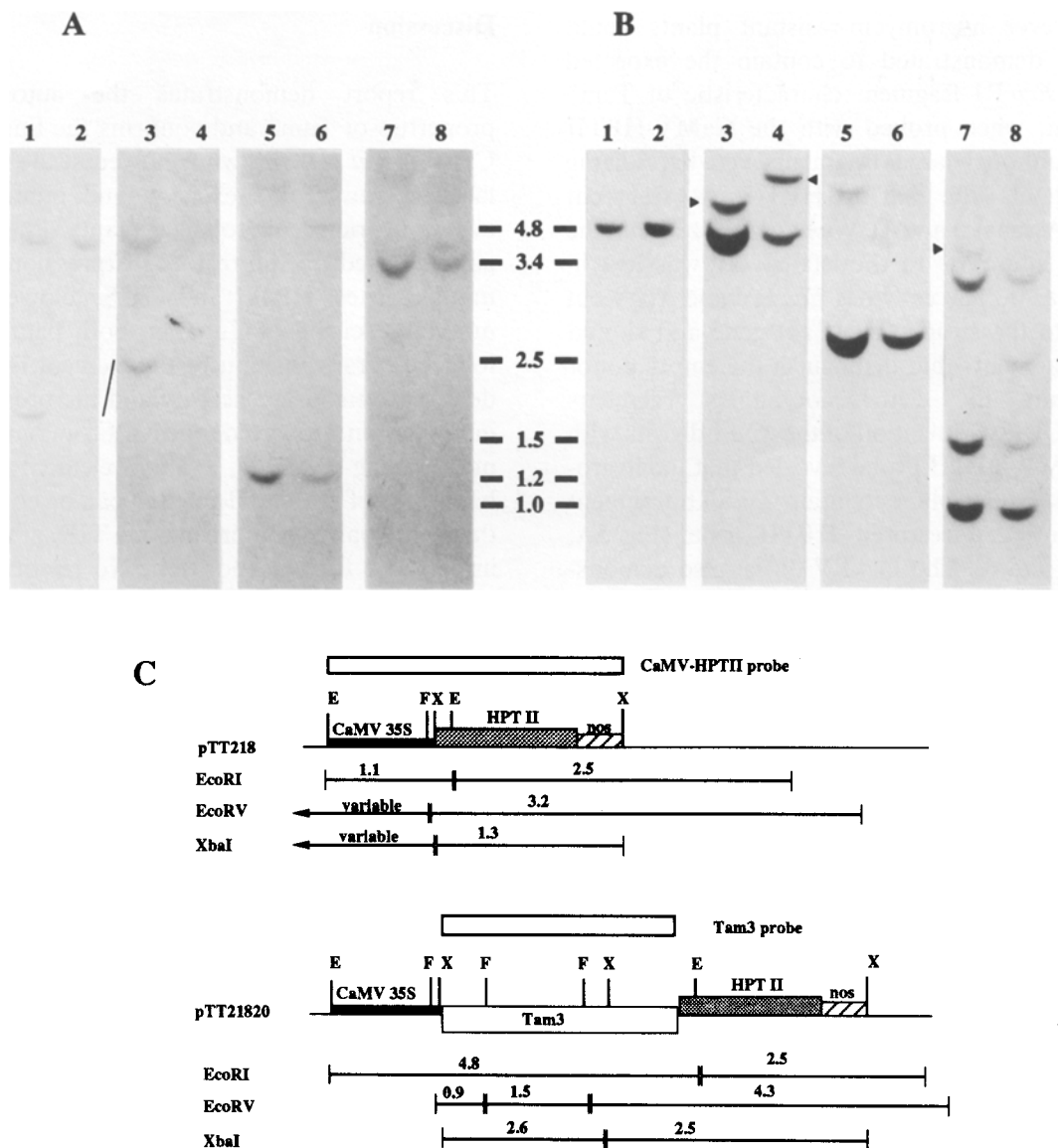


Fig. 5. Molecular analysis of Tam3 excision. 10 μ g total DNA from 2 different pTT21820-transformed plants, with a hygromycin sensitive phenotype was digested with *Eco* RI (lane 1 and 2) and DNA from 2 different plants with a resistant phenotype was digested with *Eco* RI (lanes 3 and 4), *Xba* I (lanes 5 and 6) and *Eco* RV (lanes 7 and 8), run on a 1.2% agarose gel and blotted onto Hybond-N membrane. Part A reveals the hybridization of the CaMV-HPTII-nos fragment (as described in C) to the filter, while the results of the hybridization to the Tam3 probe (as described in C) is shown in panel B. Fragment sizes of a molecular weight marker are in kilobase pairs and new Tam-3-insertions are indicated (>). The partial physical map is given in C, with the expected size of the generated fragments and the fragments used as probes.

DNA from tobacco plants transformed with pTT21820 should yield a fragment characteristic of an intact HPTII gene after excision or a larger fragment carrying Tam3, when probed with the CaMV HPTII probe (Fig. 5C). Two hygromycin-sensitive plants could be shown to yield the ex-

pected 4.8 kb *Eco* RI fragment, hybridizing to both the CaMV-HPTII probe and the Tam3 probe (Fig. 5A and B, lane 1 and 2). This implies that the hygromycin-sensitive phenotype of the transformed plant is indeed caused by the interruption of then HPTII gene by Tam3.

However hygromycin-resistant plants could not be demonstrated to contain the expected 1.1 kb *Eco* RI fragment characteristic of Tam3 excision, when probed with the CaMV-HPTII probe, although larger fragments were hybridizing as well as the 2.5 kb HPTII-nos fragment (Fig. 5A, lanes 3 and 4). We hypothesized that the *Eco* RI site close to the left border was lost in those cases. The enzymes *Eco* RV and *Xba* I cut closer to the structural HPTII gene and should yield fragments that distinguish the empty donor site from the Tam3 containing fragments (Fig. 5C). Hybridization of the *Xba* I digests with the CaMV-HPTII probe revealed that the hygromycin-resistant plants contain a 1.3 kb fragment representing a restored HPTII gene (Fig. 5A, lanes 5 and 6). The *Eco* RV digest also demonstrated the excision of Tam3 in the hygromycin-resistant plants. A 3.2 kb *Eco* RV fragment hybridizes to the CaMV-HPTII probe (Fig. 5A, lanes 7 and 8) in the hygromycin-resistant plants. Thus we conclude that the phenotypic assay for Tam3 excision and the molecular data are in agreement.

Next we addressed the question of Tam3 integration at new sites in the genome, by hybridizing the same blot with a Tam3 probe (Fig. 5B). The new bands hybridizing in the *Eco* RI digest of both hygromycin-resistant plants indicate that Tam3 might have integrated into the genome at new positions (indicated by arrowheads in Fig. 5B, lane 3 and 4). These possible Tam3-tobacco DNA fragments are also detected in the *Eco* RV digests (Fig. 5B, lanes 7 and 8). As the new fragments do not appear in the hybridization with the CaMV-HPTII-nos probe (Fig. 5A), the conclusion can be drawn that these fragments are not the result of rearrangements in pTT21820 but represent new integrations of Tam3. The high molecular weight bands in the *Xba* I digest, although too faint to be conclusive, also support the idea of new Tam3 integration. Still, the two resistant plants analysed have either a chimaeric character or contain two T-DNA inserts, since the original 4.8 kb *Eco* RI and 4.3 kb *Eco* RV fragments originating from pTT21820 (Fig. 5C) also hybridize to the Tam3 probe.

Discussion

This report demonstrates the autonomous properties of Tam3 and confirms the findings of C. Martin *et al.* [36], who showed at the molecular level that Tam3 excision and reintegration occurs in transgenic tobacco plants. Phenotypic assays based on the restored expression of two marker genes, HPTII and GUS, allowed us to monitor excision of Tam3 in both petunia and tobacco. Transposition of the element is dependent on transposase activity and the presence of intact terminal inverted repeats. From our experiments using pVUT62, a Tam3 element with 24 base pairs of one end deleted, it can be concluded that these bases, comprising one TIR, are of vital importance for transposition. No restoration of marker gene expression was detected, in a total of 187 calli and shoots analysed, when this element was present in the leader sequence. Further experiments to delimit the minimal repeat sequence required for transposition can now be initiated using this phenotypic assay system.

When the frequencies of marker gene restoration in pTT21820 and pTT224 (Tables 1 and 2) are normalized and corrected for the 65–70% expression of both markers in pTT218 and pBI121, the excision frequency can be estimated [2]. We estimated that the frequency of Tam3 excision lies between 20 and 66% in tobacco and between 18 and 40% in petunia, depending on the assay used (Table 1). The application of the GUS gene as a phenotypic reporter indicated a lower frequency of excision for Tam3 in both petunia and tobacco (see Table 1A and B).

There are several explanations possible for these differences; one is the fact that the GUS assay, performed in micro-quantities using an UV illuminator, is not sensitive enough to detect low levels of activity which are the result of position effects or variegation in a transformed leaf. Because of the long tissue culture period between transformation and the actual determination of the phenotype, excision can occur at any time, which might lead to chimaeric tissues. Indeed, we detected variegation as a result of somatic excision in a callus transformed with pGT153. The callus

gave rise to shoots with no GUS activity and shoots with different levels of enzyme activity. The other explanation might be that the GUS reporter values represent the *in vivo* situation, because transposition of the element could be induced in cells of calli that were severely stressed by the dying, hygromycin-sensitive, cells surrounding it. An enrichment of resistant parts in calli under selection has already been proposed by Baker *et al.* [2].

The transposition frequency of Ac in tobacco (17–36%) is in agreement with the data from published phenotypic assays [2] and analysis of the DNA of transgenic plants containing the element [30], but slightly below the values reported from phenotypic assays with *Solanum tuberosum* [21] and DNA analysis in *Lycopersicon esculentum* [35], and our unpublished results. This allows the conclusion that leaf disc transformation, combined with a phenotypic assay based on restored hygromycin resistance can be applied to determine the capacity of transposable elements to excise in different plant species.

When the selection level of hygromycin is lowered to 20 mg/l, the number of hygromycin-resistant calli, as a result of pTT218 and pTT224 transformation, increases (Table 2). This increase in frequency probably illustrates the influence of T-DNA position in the plant genome on the expression of the marker gene. A similar increase (+8%) was found when hygromycin-sensitive pTT21820-derived tobacco calli, containing Tam3, were rescreened on medium containing 20 mg/l hygromycin, although this increase might reflect delayed excision events during the four weeks of culture on kanamycin-containing medium. The differences in the Tam3 excision frequencies in petunia and tobacco, which are only recorded with the hygromycin reporter (not with the GUS reporter, see Table 1A and B), probably reflect the different sensitivity for hygromycin of these two plant species. We concluded that the detection of transposon excision from the HPTII gene is dependent on the level of selection that is applied and should therefore be adjusted for any other plant species tested.

When hygromycin selection was used for the

detection of Tam3 excision in tobacco, a higher frequency (Table 1A) was found as compared to that of Ac (Table 2). These results might be explained by the different origins of the elements used, but on the other hand it might as well be the result of a Tam3 cassette with a less interfering flanking sequence. The 60 bp of *waxy* sequence flanking Ac are known to interfere with NPTII gene expression in tobacco [2], but the 48 bp of *pallida* sequence remaining after excision might not have this effect.

The actual excision has been demonstrated by Southern blot analysis. Five hygromycin-resistant plants transformed with pTT224 all contained a 1.1 kb *Eco* RI fragment, representing a restored promoter-HPTII gene fusion as a result of Ac excision (Fig. 4A). No fragments were detected with the CaMV probe that indicated that some of the Ac copies still reside in the HPTII gene, as Baker *et al.* [2] observed in the transformed calli they analyzed. After hybridization with Ac, new bands (Ac-tobacco flanking sequences) differing in size in each transformant appeared (Fig. 4B). However in two plants no distinct bands could be detected, implying that Ac does not necessarily integrate after excision. But it could also be reasoned that a high somatic excision frequency of Ac results in many new border fragments generating a smear of hybridizing bands. Still both features necessary for an effective transposon tagging system are present: Ac excision correlates with the restored marker gene expression and in the majority of the cases Ac reintegrates at new positions in the genome of the host plant.

Tam3 excision in drug-resistant plants transformed with pTT21820 is clearly demonstrated by the hybridization of the 1.3 kb *Xba* I fragment to the CaMV-HPTII-nos probe (Fig. 5A). This fragment only occurs in resistant plants and results from excision of Tam3. Additional evidence comes from the *Eco* RV digestion of plant DNA, because these resistant plants contain the expected 3.6 kb *Eco* RV fragment. Hybridization of the blots with a Tam3 probe reveals that the two resistant plants analysed have either a chimaeric character or contain two T-DNA inserts. Both

the Tam3-interrupted and a new Tam3-tobacco DNA fragments hybridize to Tam3 (Fig. 5B). As the hybridization of the *Xba* I digests with the CaMV probe (data not shown) yields two border fragments in these cases, we concluded that at least two copies of the T-DNA have integrated into the plant genome and that those two plants are not chimaeric. Excision of one of the two Tam3 copies is obviously sufficient to result in a hygromycin-resistant plant. The evidence that the new fragments are composed of tobacco DNA and Tam3 and not of rearranged pTT21820 sequences is provided by the lack of hybridization of these fragments to the CaMV-HPTII-nos probe (Fig. 5A). These molecular data confirm that Tam3, like Ac, can reintegrate after excision.

Transactivation of inactive, Ds-like, elements has helped in elucidating transposition factors and could be useful for controlled transposon tagging [32]. The deleted forms of the transposable elements Tam3 and Ac might be used to set up artificial two-element systems. Therefore we initiated transformation experiments with tobacco plants containing a Tam3 element with part of the transposase coding region replaced by a plasmid (pTT21840, Fig. 2A). Leaf discs were transformed with an *Agrobacterium* strain containing plasmid pAJ4 ([24] pBIN19, with functional Tam3 in *pallida*, contains no HPTII gene). This resulted in hygromycin-resistant calli, implying excision of the Ds-type Tam3 element, while in control experiments with an *Agrobacterium* strain containing plasmid pGT114 (Δ transposase) no hygromycin-resistant calli were obtained (M. Haring *et al.*, manuscript in preparation). These preliminary results indicate that transactivation of a Ds-like Tam3 element can be achieved with the transposase function of an intact Tam3 element. The transposon Tam3 transposes with high frequency in transgenic plants and might be more suitable for tagging than Ac. Modulation of the transposition process might be possible because Tam3 is known to excise in *A. majus* with a 1000-fold higher frequency at 15 °C than at 25 °C [24]. Experiments are in progress to determine whether this temperature effect also occurs in transgenic plants. Especially

for transposon tagging experiments this might be very useful because excision will be dependent on the presence of an activating Tam3 transposase function and might be regulated by a temperature shift. The Ds-like Tam3 element used in this system is equipped with an *E. coli* plasmid, which will allow direct recloning of plant target sequences.

Acknowledgements

The authors wish to thank Dr C. Martin, Norwich for making the pAJ-4 clone and the physical map available to us and for access to the manuscript prior to publication; Dr P. Starlinger, Cologne for the Ac-containing plasmid pJAC; José Veken and Robert Windrich for skilful assistance in plant transformation experiments; Andrea Prescott for critical reading of the manuscript; and Anton Gerats for providing us with *Petunia* Mitchell plants. M.H. was financially supported by the Dutch Programme Committee for Biotechnology.

References

1. Baker B, Schell J, Lörz H, Fedoroff N: Transposition of the maize controlling element 'Activator' in tobacco. *Proc Natl Acad Sci USA* 83: 4844–4848 (1986).
2. Baker B, Coupland G, Fedoroff N, Starlinger P, Schell J: Phenotypic assay for excision of the maize controlling element Ac in tobacco. *EMBO J* 6: 1547–1554 (1987).
3. Baulcombe DC, Saunders GR, Bevan MW, Mayo MA, Harrison BD: Expression of biologically active viral satellite RNA from the nuclear genome of transformed plants. *Nature* 321: 446–449 (1986).
4. Behrens U, Fedoroff N, Laird A, Muller-Neumann M, Starlinger P, Yoder J: Cloning of *Zea mays* controlling element Ac from the *wx-m7* allele. *Mol Gen Genet* 194: 346–347 (1984).
5. Carpenter R, Martin C, Coen ES: Comparison of genetic behaviour of the transposable element Tam3 at two unlinked pigment loci in *Antirrhinum majus* *Mol Gen Genet* 207: 82–89 (1987).
6. Cone KC, Burr FC, Burr B: Molecular analysis of the maize anthocyanin regulatory locus *C1*. *Proc Natl Acad Sci USA* 83: 9631–9635 (1986).
7. Day A, Schirmer-Rahire M, Kuchka MR, Mayfield SP, Rochaix J-D: A transposon with an unusual arrangement of long terminal repeats in the green alga *Chlamydomonas reinhardtii*. *EMBO J* 7: 1917–1927 (1988).

8. Dellaporta SL, Woods J, Hicks JB: A plant DNA mini-preparation version II. *Plant Mol Biol Rep* 1(4): 19–21 (1983).
9. De Vlamming P: Genotype determination of *Petunia* Mitchell with respect to some flower color genes. *Plant Mol Biol News* II: 106–108 (1981).
10. Ditta G, Stanfield S, Corbin D, Helinski DR: Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci USA* 12: 7347–7351 (1980).
11. Fedoroff NV, Furtek DB, Nelson OE Jr: Cloning of the *bronze* locus in maize by a simple and generalizable procedure using the transposable controlling element *Activator* (*Ac*). *Proc Natl Acad Sci USA* 81: 3825–3829 (1984).
12. Gielen J, De Beuckeleer M, Seurinck J, Deboeck F, De Greve H, Lemmers M, Montagu M van, Schell J: The complete nucleotide sequence of the TL-DNA of the *Agrobacterium tumefaciens* plasmid pTiAch5. *EMBO J* 3: 835–846 (1984).
13. Goddard JM, Caput D, Williams SR, Martin DW: Cloning of human purine-nucleoside phosphorylase cDNA sequences by complementation in *Escherichia coli*. *Proc Natl Acad Sci USA* 80: 4281–4285 (1983).
14. Grandbastien M-A, Spielmann A, Caboche M: Tnt-1 a mobile retroviral like transposable element of tobacco isolated by plant cell genetics. *Nature* 337: 376–380 (1989).
15. Herrmann A, Schulz W, Hahlbrock K: Two alleles of the single-copy chalcone synthase gene in parsley differ by a transposon-like element. *Mol Gen Genet* 212: 93–98 (1988).
16. Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA: A binary vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* 303: 179–180 (1983).
17. Hudson A, Carpenter R, Coen ES: De novo activation of the transposable element Tam2 of *Antirrhinum majus*. *Mol Gen Genet* 207: 54–59 (1987).
18. Inzé D, Follin A, Lijsebettens M van, Simoens C, Genetello C, Montagu M van, Schell J: Genetic analysis of the individual T-DNA genes of *Agrobacterium tumefaciens*; further evidence that two genes are involved in indole-3-acetic acid synthesis. *Mol Gen Genet* 194: 265–274 (1984).
19. Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6: 3901–3907 (1987).
20. Jones JDG, Dunsmuir P, Bedbrook J: High level expression of introduced chimaeric genes in regenerated transformed plants. *EMBO J* 4: 2411–2418 (1985).
21. Knapp S, Coupland G, Uhrig H, Starlinger P, Salamini F: Transposition of the maize transposable element *Ac* in *Solanum tuberosum*. *Mol Gen Genet* 213: 285–290 (1988).
22. Maliga P, Breznovitz A, Marton L: Streptomycin resistant plants from callus culture of resistant tobacco. *Nature* 244: 29–30 (1973).
23. Maniatis T, Fritsch EF, Sambrook J: *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
24. Martin C, Carpenter R, Sommer H, Saedler H, Coen ES: Molecular analysis of instability in flower pigmentation of *Antirrhinum majus*, following isolation of the *pallida* locus by transposon tagging. *EMBO J* 4: 1625–1630 (1985).
25. Motto M, Maddaloni M, Ponziani G, Brembilla M, Marotta R, Di Fonzo N, Soave C, Thompson R, Salamini F: Molecular cloning of the *o2-m5* allele of *Zea mays* using transposon marking. *Mol Gen Genet* 212: 488–494 (1988).
26. Nevers P, Shepard N, Saedler H: Plant transposable elements. *Adv Bot Res* 12: 103–203 (1986).
27. O'Reilly C, Shepherd NS, Pereira A, Schwarz-Sommer Z, Bertram I, Robertson DS, Peterson PA, Saedler H: Molecular cloning of the *al* locus of *Zea mays* using the transposable elements *En* and *Mul*. *EMBO J* 4: 877–882 (1985).
28. Paz-Ares J, Wienand U, Peterson PA, Saedler H: Molecular cloning of the *c* locus regulating the anthocyanin pathway. *EMBO J* 5: 829–833 (1986).
29. Sanger F, Nicklen S, Coulson R: DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* 74: 5463–5467 (1980).
30. Shirsat AH: A transposon-like structure in the 5' flanking sequence of a legumin gene from *Pisum sativum*. *Mol Gen Genet* 212: 129–133 (1988).
31. Sommer H, Carpenter R, Harrison BJ, Saedler H: The transposable element Tam3 of *Antirrhinum majus* generates a novel type of sequence alterations upon excision. *Mol Gen Genet* 199: 225–231 (1985).
32. Sutton WD, Gerlach WL, Schwartz D, Peacock WJ: Molecular analysis of Ds Controlling element mutations at the *Adhl* locus of maize. *Science* 233: 1265–1269 (1984).
33. Van Sluys MA, Tempé J, Fedoroff N: Studies on the introduction and mobility of the maize *Activator* element in *Arabidopsis thaliana* and *Daucus carota*. *EMBO J* 6: 3881–3889 (1987).
34. Vodkin LO, Rhodes PR, Goldberg RB: A Lectin gene insertion has the structural features of a transposable element. *Cell* 34: 1923–1931 (1983).
35. Yoder JJ, Palys J, Alpert K, Lassner M: *Ac* transposition in transgenic tomato plants. *Mol Gen Genet* 213: 291–296 (1988).
36. Martin C, Prescott A, Lister C, MacKay A: Activity of the transposon Tam3 in antirrhinum and tobacco: possible role of DNA methylation *EMBO J* 8: 997–1004 (1989).